

## AN ABSTRACT OF THE THESIS OF

William L. Gale for the degree of Master of Science in Fisheries Science presented on March 11, 1996. Title: Sexual Differentiation and Steroid-Induced Sex Inversion in Nile Tilapia (*Oreochromis niloticus*): 1. Characterization of a Gonadal Androgen Receptor. 2. Masculinization by Immersion in Methylidihydrotestosterone.

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Martin S. Fitzpatrick and Carl B. Schreck

Steroids control gonadal function and development in fishes. They are thought to be the natural inducers of gonadal differentiation, controlling whether the indifferent gonad develops as an ovary or a testis. When administered to differentiating fry, androgens induce testicular development and estrogens initiate ovarian development. This process, called steroid-induced sex inversion, is used in commercial aquaculture and laboratory studies. The goal of this thesis was to increase the efficiency of steroid-induced sex inversion and to understand the molecular mechanism of gonadal differentiation and steroid-induced sex inversion.

To better understand the molecular mechanism of androgen effects on the gonads, the binding of the synthetic androgen  $^3\text{H}$ -mibolerone was characterized in gonadal tissue of Nile tilapia (*Oreochromis niloticus*). Binding is described for adult males and immature females. Testicular ( $K_d=1.0\pm0.1$  nM,  $B_{\max}=35.8\pm1.2$  fmol/mg protein;  $n=3$  assays) and ovarian ( $K_d=0.30\pm0.06$  nM,  $B_{\max}=14.6\pm1.1$  fmol/mg protein;  $n=3$  assays) binding sites displayed high affinity and low capacity for mibolerone binding. Scatchard

plots of transformed saturation data were linear, indicating a single class of binding site. The receptor was predominately found in gonadal cytosol, with some specific binding observed in liver and heart cytosol. Only androgens and steroids with sex inverting capacities displaced tritiated mibolerone, thus the receptor demonstrated ligand binding specificity. Furthermore, the receptor had DNA binding affinity, as measured by adherence to calf thymus DNA-cellulose. Kinetic characteristics of testicular binding sites differed from ovarian binding sites. These discrepancies may reflect sex related differences in androgen receptor function and regulation. If this receptor is present in differentiating fry, it may be responsible for signal transduction during gonadal differentiation and steroid-induced sex inversion. Involvement in other processes such as spermatogenesis and gonadal growth are also possible.

The efficacy of a short term immersion procedure for masculinizing Nile tilapia was examined. Two synthetic androgens were evaluated, methyl dihydrotestosterone and 17 $\alpha$ -methyltestosterone. Three hr exposures of fry at 10 and 13 days post-fertilization in methyl dihydrotestosterone at 500  $\mu$ g/l produced sex ratios greater than 93 percent male. Immersions in methyl dihydrotestosterone at 100  $\mu$ g/l and methyltestosterone at 500 or 100  $\mu$ g/l were unsuccessful at producing all-male populations. Immersion of Nile tilapia in 500  $\mu$ g/l methyl dihydrotestosterone may provide a practical alternative to the use of steroid-treated feed. Furthermore, when compared with current techniques for steroid-induced sex inversion of tilapia, short term immersion shortens the treatment period and reduces the risk of worker exposure to anabolic steroids.

Sexual Differentiation and Steroid-Induced Sex Inversion in Nile Tilapia (*Oreochromis niloticus*): 1. Characterization of a Gonadal Androgen Receptor.  
2. Masculinization by Immersion in Methylidihydrotestosterone.

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William L. Gale, Author

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## **Contribution of Authors**

Dr. Martin S. Fitzpatrick and Dr. Carl B. Schreck were involved in the design and analysis of all experiments described in Chapter 1 and 2.

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Sexual Differentiation and Steroid-Induced Sex Inversion in Nile Tilapia (*Oreochromis niloticus*): 1. Characterization of a Gonadal Androgen Receptor.  
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## **Introduction**

The wealth of sexual differences present in the animal kingdom has always intrigued and mystified scientists. In the past century, biologists have slowly elucidated the genetic and biochemical basis for vertebrate sex determination and gonadal differentiation. In mammals these processes are well defined (see reviews by Nagai, 1992 and Schafer, 1995). However, in the remaining vertebrate groups, sex determination and gonadal differentiation are, at best, partially understood. Jost (1972 as cited by Nagai, 1992) proposed that sex determination consists of a sequential progression of events beginning at the time of fertilization. Each step in the process is dependent on and controlled by the preceding one. Genetic sex is initially determined at the time of fertilization. The genetic (chromosomal) characteristics control gonadal sex differentiation, which subsequently directs development of secondary sex characteristics. In most vertebrates, this paradigm of sexual development has held true. However, environmental cues are also important regulators of gonadal differentiation and secondary sex characteristics (e.g., temperature dependent sex determination in some reptiles and fishes).

## Genetic and Biochemical Basis of Gonadal Differentiation in Mammals

### *Genetic basis of gonadal differentiation.*

A monofactorial system governs mammalian sex determination. In normal development, presence of a karyotypically distinct male chromosome (Y) is required for male development (except in cases of fragment translocation) and ovarian development occurs in the absence of a Y chromosome. The region of the Y chromosome that initiates testicular development, called Sry (Sinclair *et al.*, 1990; Gubbay *et al.*, 1990), seems to encode a transcription factor-like DNA-binding protein. Lacking Sry, the bipotential mammalian gonad develops into an ovary (see review by Nagai, 1992).

### *Biochemical basis for differentiation of gonadal anlage.*

The genetic mechanism of male sex determination triggers a regulatory cascade with three biochemical outcomes that, when combined, facilitate testicular differentiation (see reviews by Nagai, 1992 and Schafer, 1995). Testosterone, produced by the Leydig cells, virilizes the internal and external genitalia. Anti-Müllerian hormone, a glycopeptide produced in the Sertoli cells, causes regression of the Müllerian ducts. Lastly, Sry inhibits aromatase, an enzyme that facilitates conversion of testosterone to estrogen (Haqq *et al.*, 1993).

Ovarian differentiation proceeds in the absence of Sry, and is thought to be the default plan of gonadal development. The genes responsible for ovarian differentiation and their biochemical products have not been fully identified.

### Genetic and Biochemical Basis of Gonadal Differentiation in Fishes

#### *Genetic basis of gonadal differentiation.*

Fishes display a variety of strategies for the genetic determination of sex, ranging from strict monofactorial systems to polygenic mechanisms of sex control. Salmonids, for example, possess a stable monofactorial system in which males are heterogametic (XY), and females are homogametic (XX) (see review by Tave, 1986). Conversely, certain other fish species possess a stable monofactorial system in which, females are heterogametic (ZW) and males are homogametic (ZZ) (e.g., *Anguilla japonica*, *Gambusia affinis*, and *Leporinus obtusidens*; as reviewed by Solari, 1994). Sex determination in tilapia was long thought to be monofactorial in nature (Clemens and Inslee, 1968); however, Mair *et al.* (1991a,b) recently demonstrated that sex determination deviates from a strict monofactorial system in blue tilapia (*Oreochromis aureus*) and in Nile tilapia (*O. niloticus*). Autosomal and environmental influences have been proposed to explain this deviation (Mair *et al.*, 1991a, 1991b; Trombka and Avtalion, 1993). Lastly, in a small group of gonochoristic teleosts (e.g., *Xiphorus helleri*, *Poecilia vittata*, *P. caudofasciata*), a polygenic system of sex determination is present (see reviews by Yamamoto, 1969; Chan and Yeung, 1983; Solari, 1994). Furthermore, environmental factors can be important effectors of polygenic systems of sex determination. For example, in the Atlantic silverside (*Menidia menidia*), exposure to low temperature results in a skewing of the sex ratio toward female (Conover and Fleisher, 1986). Since,

the response of fry to temperature exposure is highly dependent on parental lineage, environmental cues are thought to be under genetic control in the silverside (Conover and Heins, 1987). In certain cichlid species (*Pelvicachromis*, *Apistogramma* spp.), pH is an important controller of sex determination (Rubin, 1985). Rubin observed a significant skewing of the sex ratio towards males in response to low pH. Conversely, exposure to a high pH caused skewing of the sex ratio towards females.

*Biochemical basis of gonadal differentiation.*

Many researchers have described the histological events of testicular and ovarian differentiation in fish. However, few correlations have been made between histological events and the biochemical process of gonadal differentiation. These correlations are difficult to make given the lack of knowledge concerning the biochemical initiation of gonadal differentiation in fish. This problem is further complicated by the difficulties inherent in working with differentiating fry. Their small size makes it nearly impossible to dissect out individual tissues, and it is even more difficult to understand biochemical events at the cellular level.

After making observations of the sex-inverting effect of steroids on medaka (*Oryzias latipes*), Yamamoto (1969) proposed that steroids are the natural inducers of gonadal differentiation in fish. Decisive evidence refuting Yamamoto's hypothesis has not been found, and much circumstantial evidence in support of his theory has been reported. Feist *et al.* (1990) demonstrated that coho salmon (*Oncorhynchus kisutch*) can synthesize testosterone before gonadal differentiation. The site of testosterone synthesis is

unknown, but this steroid may play an important role in gonadal differentiation.

Fitzpatrick *et al.* (1993) found that interrenal tissue of rainbow trout (*O. mykiss*) can produce androstenedione (*in vitro*) at the time of gonadal differentiation, and suggested that this tissue may be a site of production for masculinizing hormones. Nakamura and Nagahama (1985, 1989) described (histologically) the presence of steroid-producing cells before gonadal differentiation in the Nile tilapia. A comprehensive examination of steroid production in the developing tilapia has not been done; however, the presence of steroid-producing cells prior to histological differentiation suggests a natural role for steroids in gonadal differentiation of tilapia.

Several naturally-occurring steroids have masculinizing or feminizing capabilities.  $17\beta$ -Estradiol when administered to differentiating salmonids results in a significant skewing of sex ratios in favor of females (Goetze *et al.*, 1979; Piferrer and Donaldson, 1989). Furthermore, the naturally-occurring androgens  $11\beta$ -hydroxyandrostenedione and 11-ketotestosterone have masculinizing effects. Implantation of 11-ketotestosterone results in the proliferation of testicular tissue in ovariectomized goldfish (*Carassius auratus*; Kobayashi *et al.*, 1991), and administration of  $11\beta$ -hydroxyandrostenedione to differentiating rainbow trout results in masculinization (van den Hurk and van Oordt, 1985; Feist *et al.*, 1995). These results further support the proposed involvement of steroids in gonadal differentiation.

A vast array of synthetic steroidal compounds masculinize or feminize differentiating fry (see reviews by Schreck, 1974 and Hunter and Donaldson, 1983). Interestingly,

masculinizing potential is generally associated with synthetic 17-alkylated androgens, such as 17 $\alpha$ -methyltestosterone, methyl dihydrotestosterone, mibolerone, and fluoxymesterone. Paradoxical feminization by androgen treatment has been noted in several fish species (Hunter and Donaldson, 1983), and is probably caused by aromatization of androgen to a feminizing estrogen compound. In coho salmon, the use of nonaromatizable androgen (methyl dihydrotestosterone) avoided this phenomenon (Piferrer and Donaldson, 1991). Hines and Watts (1995) reported the masculinizing potential of two non-steroidal compounds, acroflavin and tamoxifen (in Nile tilapia); however, the mechanism by which these compounds exert their influence is unknown.

Several synthetic estrogens feminize differentiating fry. Diethylstilbesterol and 17 $\alpha$ -ethynylestradiol are the most widely used of these compounds. The naturally occurring steroid 17 $\beta$ -estradiol has also been used as a feminizing agent (Goetze, 1979 and Piferrer and Donaldson, 1989). Although estradiol is a potent feminizing agent in many fish species (see reviews by Schreck, 1974 and Hunter and Donaldson, 1983) it has little effect on tilapia (*Oreochromis* spp.: Jensen, 1976; Hopkins *et al.*, 1979; and Rosenstein and Hulata, 1992).

The biochemical mechanism of steroid-induced sex inversion is not fully understood. In some fish species, the site of accumulation and the clearance mechanism for sex-inverting compounds has been described. In Nile tilapia, radiolabelled methyltestosterone fed to differentiating fry was largely confined (>90%) to the visceral tissue (Goudie *et al.*, 1986). Similar findings were found in Mozambique tilapia (*O.*



*mossambicus*) and rainbow trout (Johnstone *et al.*, 1983). Since the gonads were considered as part of the viscera, these results suggest that masculinizing steroids have a direct effect on the gonads rather than via effects on the pituitary gonadal axis.

### Conclusions

The biochemical events leading to gonadal differentiation are incompletely understood in fish. Given the potent effects of steroids on the gonads, Yamamoto's hypothesis that steroids are the natural inducers of sex differentiation must still be considered. If androgens direct testicular development and estrogens direct female development, then control of aromatase may be key in initiating gonadal differentiation. In the mammalian system, Sry initiates testosterone production by the Leydig cells and inhibits aromatase activity. A similar system of genetic control may be present in gonochoristic fishes. The control of ovarian differentiation is incompletely understood in mammals. Circumstantial evidence supports the role of separate X-linked genes in initiating mammalian ovarian development (reviewed by Schafer, 1995). The specific gene(s) controlling ovarian differentiation in fish has not been identified.

Given the evidence supporting a direct effect of steroids on gonadal differentiation, it is likely that specific steroid receptors are present in the indifferent gonads that facilitate steroid-induced sex inversion and natural gonadal differentiation. The standard model of steroid action involves binding to and activation of a highly specific intracellular receptor. The activated receptor/steroid complex acts as a transcription factor, interacting with specific response elements on the DNA (Carson-Jurica *et al.*, 1990; O'Malley and

Tsai, 1992). Fitzpatrick *et al.* (1994) characterized an androgen receptor in the ovaries of juvenile coho salmon. This receptor was specific for sex-inverting steroids and may represent persistence of the molecule responsible for sexual differentiation. Additional research on the presence of steroid receptors in the gonads of teleost fish has not been done.

A combined approach of basic and applied research methods was used in this thesis. The former approach sought to better understand the molecular mechanism of gonadal differentiation and steroid-induced sex inversion, by examining the binding of the masculinizing androgen mibolerone to Nile tilapia gonadal tissue. The results of this research are described in Chapter 1. This is the first examination of androgen receptors in tilapia (*Oreochromis* spp.), and only the second description of gonadal androgen receptors in teleosts. If present in differentiating fry, this receptor may represent persistence of the molecule mediating gonadal differentiation and steroid-induced sex inversion. The goal of the applied research was to increase the efficiency of steroid-induced sex inversion. Chapter 2 presents the first successful short-term immersion procedure for masculinization of Nile tilapia. Masculinization by short-term immersion in steroid solution shortens the period of treatment while reducing the risk of worker exposure to anabolic steroids.

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## Chapter 1

### **Binding Characteristics of a Gonadal Androgen Receptor in Nile Tilapia (*Oreochromis niloticus*)**

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### Abstract

An androgen receptor in the testes and ovaries of Nile tilapia (*Oreochromis niloticus*) was characterized using the synthetic androgen mibolerone (17-hydroxy-7,17-dimethyl-4-en-3-one). Both the testicular ( $K_d=1.0\pm0.1$  nM,  $B_{max}=35.8\pm1.2$  fmol/mg protein;  $n=3$ ) and ovarian ( $K_d=0.30\pm0.06$  nM,  $B_{max}=14.6\pm1.1$  fmol/mg protein;  $n=3$ ) binding sites displayed high affinity and low capacity for mibolerone binding. Scatchard plots of the transformed saturation data were linear, indicating a single class of binding site. The receptor was predominately found in gonadal cytosol, with some specific binding observed in liver and heart cytosol. Only androgens and steroids with sex inverting capacities displaced tritiated mibolerone, thus, the receptor demonstrated ligand binding specificity. Furthermore, the receptor has DNA binding affinity, as measured by adherence to calf thymus DNA-cellulose. The kinetic characteristics of the testicular binding sites differed from those of the ovarian binding sites in that ovarian binding had higher affinity and fewer sites. These discrepancies may reflect sex related differences in androgen receptor function and regulation. If this receptor is present in differentiating fry, it may be responsible for signal transduction during sex differentiation and steroid-induced sex inversion. Involvement in other processes such as spermatogenesis and gonadal growth is also possible.



### Introduction

Androgens potently control reproduction in fish through their involvement in such diverse physiological processes as sexual differentiation, gametogenesis, spawning behavior, and expression of secondary sex characteristics. The endocrine effects of androgens on peripheral tissues (e.g., skin and fins) and on behavior is well known (Liley and Stacey, 1983). Conversely, little is known about the autocrine and paracrine effects of androgens on the gonads. These local effects are critical to the reproductive success of the animal, and androgens are thought to play key roles in both sexual differentiation and spermatogenesis. Steroidal messengers require the presence of specific intracellular receptors that facilitate the influence of steroids on gene expression. Therefore, receptors must be present in the gonads of fish that facilitate the autocrine and paracrine effects of androgens.

Spermatogenesis in fish is thought to be androgen-dependent (Billard *et al.*, 1982, Callard, 1992). In the spiny dogfish shark (*Squalus acanthias*), androgen sensitivity (as measured by maximal androgen receptor number) is observed in testicular cells before or during early spermatogenesis (Cuevas and Callard, 1992). Furthermore, administration (*in vitro*) of androgens to nonreproductive testes induces spermatogenesis in the Japanese eel (*Anguilla japonica*; Miura *et al.*, 1991) and the mummichog (*Fundulus heteroclitus*; Cochran, 1992). Clearly, androgens have at least one local effect on testicular function, however, autocrine or paracrine effects in addition to spermatogenesis have yet to be demonstrated.

Aquaculturists and researchers commonly use androgens to direct sexual differentiation in fish (see reviews in Schreck, 1974; Hunter and Donaldson, 1983). Based on observations of the sex-inverting effect of steroids in the medaka (*Oryzias latipes*), Yamamoto (1969) hypothesized that androgens are the natural inducers of gonadal differentiation. Definitive evidence supporting this hypothesis has yet to be found. Feist *et al.* (1990) found that coho salmon (*Oncorhynchus kisutch*) can synthesize testosterone (T) before the onset of histological sex differentiation. Furthermore, Nakamura and Nagahama (1985, 1989) described the presence of steroid producing cells in gonads of histologically differentiating Nile tilapia (*Oreochromis niloticus*) fry. The molecular mechanism by which naturally-occurring steroids might direct sexual differentiation is not understood.

In vertebrates, androgens act via binding to and activation of a highly specific steroid receptor. The androgen receptor (AR) is a ligand-dependent transcription factor that transactivates protein expression by binding to and interacting with response elements on the DNA (Carson-Jurica *et al.*, 1990; O'Malley and Tsai, 1992). Clark and Peck (1977) proposed that putative steroid receptors should meet a minimum set of criteria based on the accepted model of steroid action. A steroid receptor should bind to a specific class of steroids, have high affinity for the ligand, be present in low concentration, be localized in a specific tissue or set of tissues, and be correlated with a biological response.

This model of steroid action combined with observations on the potent effects of androgens on sex differentiation in fish would necessitate the presence of an AR in

undifferentiated fish. Furthermore, the simplest explanation of masculinizing effects of androgens would be through direct effects on the gonads via an AR. Fitzpatrick *et al.* (1994) characterized an AR in the ovaries of juvenile coho salmon (*Oncorhynchus kisutch*). The receptor met all of the criteria for a model steroid receptor (Clark and Peck, 1977) and was highly specific for steroids capable of directing sexual differentiation.

The objective of this study was to determine if an AR is present in the gonads of Nile tilapia. Although our goal was to determine the link between ARs and sex differentiation, the study focused on adult males and immature females because of the difficulties in obtaining sufficient tissue from differentiating fry. Presence of an AR may not be absolutely indicative of an involvement in steroid-induced sex differentiation; however, presence of an AR in the gonads is necessary if androgens act directly on the gonads to affect sex differentiation. The existence of ARs in differentiated gonads may reflect persistence of the molecule responsible for mediating steroid-induced sex inversion. In addition, in males, this putative receptor may also be involved during spermatogenesis and testicular growth (i.e., anabolic effects).

### Materials and Methods

#### *Animals and tissue preparation.*

Nile tilapia (Ivory Coast Strain) were reared in recirculating systems, which included filters for particle separation and ammonia conversion, at Oregon State University,

Corvallis, Oregon. Temperature was maintained at 24-29°C. Fish were fed to satiation twice daily with either a commercial catfish diet or a commercial trout diet.

Cytosolic fractions were prepared from mature testes, mature and immature ovaries, liver, white muscle, heart, and gill samples. Cytosol and nuclear fractions were prepared as described by Fitzpatrick *et al.* (1994). In brief, fish were killed by overdose of anesthetic (tricaine methanesulfonate, 300 mg/l) buffered with sodium bicarbonate. Tissues were dissected and homogenized with a motor driven Teflon homogenizer in a 2X vol buffer (TEMS; 10 mM Tris-HCl, 1mM EDTA, 20 mM Na<sub>2</sub> molybdate, 12mM  $\alpha$ -Monothioglycerol, and 10% glycerol (v/v), pH=7.4). The homogenate was centrifuged at 1500 x g for 20 min at 4°C, and the resulting pellet held on ice for extraction of the nuclear fraction. Endogenous steroid was removed from the supernatant by incubating on ice for 10 min with 0.5X vol of 5.0% charcoal (w/v) and 0.5% dextran (w/v) in TEMS. The suspension was then centrifuged at 1500 x g for 25 min at 4°C. To isolate the cytosolic fraction from the mitochondrial and endoplasmic reticulum, the supernatant was centrifuged at 100,000 x g for 60 min at 4°C. The pellet for nuclear extraction (testes only) was washed three times with nuclear wash buffer (10 mM Tris-HCl, 3 mM MgCl<sub>2</sub>, 2 mM monothioglycerol, and 250 mM sucrose, pH=7.5) and extracted with extraction buffer (50 mM Tris-HCl, 1 mM Na<sub>2</sub> EDTA, 12 mM monothioglycerol, 700 mM KCl, and 30% glycerol (v/v), pH=7.5). All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Immediately after preparation, all samples were stored at -80°C.

*Conditions for binding assays.*

Protein concentrations of cytosol and nuclear fractions were determined by method of Bradford (1976). Prior to dilution with steroid, the protein concentration of cytosol was adjusted to 5 mg/ml. Mibolerone [ $17\alpha$ -methyl- $^3\text{H}$ ] ( $^3\text{H}$ -Mb) and radioinert mibolerone (Mb) were obtained from Du Pont NEN Research Products (Boston, MA); all other steroids were obtained from Sigma Chemical Company. All steroids were stored in stock solutions of 100% ethanol. Steroids were dried under  $\text{N}_2$  (g), and dissolved in TEMS for use in experiments. In all experiments, unless otherwise noted, the final volume of cytosol and steroid solutions was 0.25 ml. Unless otherwise noted, total binding was the amount of binding observed in the absence of Mb, and nonspecific binding was determined by addition of 100-fold excess Mb. Total and nonspecific binding were determined in all binding experiments. Specific binding was calculated as the difference between total and nonspecific binding.

*Association parameters.*

Equilibrium binding conditions were determined at  $17^\circ\text{C}$ . Testicular cytosol (0.15 ml) was incubated with 1 nM  $^3\text{H}$ -Mb for varying amounts of time up to 24 hr. At sampling times, the reaction was stopped by placing the incubation tubes on ice. Unbound steroid was removed by incubation with 0.50 ml 2.5% (w/v) charcoal-0.25% (w/v) dextran in TEMS for 10 min. The tubes were centrifuged at  $1500 \times g$  for 20 min at  $4^\circ\text{C}$ . The supernatant was decanted into liquid scintillation cocktail and counted on a liquid scintillation counter (counts were corrected for machine efficiency).

*Saturation analysis of gonadal cytosol and nuclear extract.*

Saturation analysis was employed to determine the binding characteristics of the steroid/binding site interaction. Mibolerone binding was examined in testicular cytosol and ovarian cytosol.  $^3\text{H}$ -Mibolerone was incubated for 12 hr with 0.15 ml of cytosol in the presence or absence of 100X Mb. Concentrations of  $^3\text{H}$ -Mb varied from 0.025 nM to 6.0 nM. The reaction was halted, unbound steroid removed, and radioactivity determined as previously described. The dissociation constant ( $K_d$ ) and the maximum number of binding sites ( $B_{\text{max}}$ ) were determined by nonlinear regression using the computer program Prism (GraphPad Software, San Diego, CA).

To determine the ability of  $^3\text{H}$ -Mb to bind to the nuclear fraction of testicular tissue, nuclear extract (0.15 ml; 2 mg protein/ml) was incubated for 12 hr at 17°C in the presence of varying concentrations of  $^3\text{H}$ -Mb (0.0625-6.0 nM). The reaction was halted, and radioactivity determined as previously described.

*Tissue specificity.*

To assess the distribution of Mb binding sites, 1 nM  $^3\text{H}$ -Mb was incubated at 17°C for 12 hr with 0.15 ml of cytosol from white muscle, liver, heart, gill, and ovaries. All tissues (except ovaries) were obtained from adult males. The reaction was stopped, and radioactivity determined as previously described.

*Metabolism of mibolerone during incubation.*

Potential metabolism of  $^3\text{H}$ -Mb during incubation with testicular cytosol was examined by thin layer chromatography (TLC). Cytosol (0.4 ml) was incubated for 12 hr at  $17^\circ\text{C}$  with 1 nM  $^3\text{H}$ -Mb. Steroid was extracted twice with 8 ml diethyl ether and dried by vacuum centrifugation. The extract was spotted onto a TLC plate (Baker-Flex silica gel IB2-F) with a  $^3\text{H}$ -Mb standard and developed once with a dichloromethane: diethyl ether (5:2) solvent system. Radiation was counted from 1-cm sections.

*Ligand specificity.*

Testicular cytosol (0.15 ml) was incubated for 12 hr at  $17^\circ\text{C}$  with 1 nM  $^3\text{H}$ -Mb and 1, 10, 100, or 500-fold excess concentration of competitor. The reaction was halted as previously described. Competitors used were: Mb; T (4-androsten- $17\beta$ -ol-3-one, T); 11-ketotestosterone (4-androsten- $17\beta$ -ol-3, 11-dione, KT);  $5\alpha$ -dihydrotestosterone ( $5\alpha$ -androstan- $17\beta$ -ol-3-one, DHT);  $17\alpha$ -methyltestosterone ( $17\alpha$ -methyl-4-androsten-3-one, MT);  $11\beta$ -hydroxyandrostenedione (androsten- $11\beta$ -ol-3, 17-dione,  $11\text{OH-An}$ );  $17\beta$ -estradiol (1,3,5(10)-estratrien-3,  $17\beta$ -diol,  $\text{E}_2$ );  $17\alpha$ -ethynylestradiol ( $17\alpha$ -ethynyl-1,3,5(10)-estratrien-3,  $17\beta$ -diol, EE); progesterone (4-pregnen-3,20-dione,  $\text{P}_4$ ); and  $17\alpha$ ,  $20\beta$ -dihydroxyprogesterone (4-pregnen- $17\alpha$ ,  $20\beta$ -diol-3-one,  $17,20\text{-DHP}$ ). Ability to displace  $^3\text{H}$ -Mb binding was expressed as relative binding affinity, i.e., the difference between total binding and binding in the presence of competitor, divided by the difference between total binding and binding seen with 500-fold excess Mb. The ligand specificity of binding sites from immature ovaries was assessed similarly using the

following competing steroids: MT; methyl dihydrotestosterone ( $17\alpha$ -methyl androstan- $17\beta$ -ol-3-one, MDHT);  $E_2$ ; and  $P_4$ .

*DNA-cellulose chromatography.*

Calf thymus DNA-cellulose was obtained from Sigma Chemical Company. DNA-cellulose (0.4 g) was suspended in 10 ml column buffer (CB; 0.01 M NaCl and 0.02% w/v bovine serum albumin in molybdate-free TEMS) placed in a polypropylene column (Bio-Rad Laboratories, Hercules, CA) and washed with an additional 8 ml CB.

Testicular cytosol (1.2 ml; 3.5 mg protein/ml cytosol) was incubated at  $4^\circ\text{C}$  for 18 hr with 5 nM  $^3\text{H}$ -Mb in the presence or absence of 500 nM Mb (total incubation vol was 1.3 ml). Following incubation, cytosol was placed on ice and protein removed by precipitation with ammonium sulfate. A 1X vol of saturated ammonium sulfate (17.5 g ammonium sulfate in 25 ml molybdate-free TEMS) was added in 0.1 ml aliquots at 1 min intervals and then allowed to incubate while stirring for 47 minutes. The precipitate was removed by centrifugation at  $10,000 \times g$  for 20 min at  $4^\circ\text{C}$ . The pellet was washed with 1 ml CB, and resuspended in 1 ml CB. Unbound steroid was removed by incubation on ice for 10 min with 1 ml of 5.0% charcoal (w/v) and 0.5% dextran (w/v) in CB. The solution was then centrifuged at  $1500 \times g$  for 25 min at  $4^\circ\text{C}$ . An aliquot (0.2 ml) of the supernatant was removed and radioactivity determined for calculation of total specific binding. The remaining supernatant was diluted to 5 ml with CB and loaded on a DNA-cellulose column.



The loaded column was incubated for 30 min at 22°C followed by 30 min at 4°C. The column was washed consecutively with 8 ml of CB, 8 ml 0.4 M NaCl, 0.02% (w/v) bovine serum albumin in molybdate-free TEMS, and 8 ml 2 M NaCl, 0.02% (w/v) bovine serum albumin in molybdate-free TEMS. Fractions (1 ml) were collected and radioactivity determined in each fraction as previously described.

## Results

### *Association parameters.*

At 17°C equilibrium binding was achieved by 4 hr, and binding remained stable up to 16 hr (Fig. 1.1). Due to the stability of binding, all subsequent incubations were performed at 17°C for 12 hr.

### *Saturation analysis.*

Saturation analysis revealed a typical hyperbolic binding curve in testicular cytosol and in ovarian cytosol from immature females (Figs. 1.2 and 1.3). The  $K_d$  for testicular cytosol was  $1.0 \pm 0.1$  nM ( $n=3$  assays using different cytosol pools) and the  $B_{max}$  was  $35.8 \pm 1.2$  fmol/mg protein ( $n=3$  assays using different pools). Ovarian cytosol had a  $K_d$  of  $0.30 \pm 0.06$  nM ( $n=3$ ) and a  $B_{max}$  of  $14.6 \pm 1.1$  fmol/mg protein ( $n=3$ ). Scatchard plots of the transformed data were linear for both ovarian and testicular cytosol (Figs. 1.2 and 1.3). Ovarian cytosol from mature females displayed little specific binding and was unsaturable (data not shown). No specific binding was observed in nuclear extract prepared from testes (data not shown).

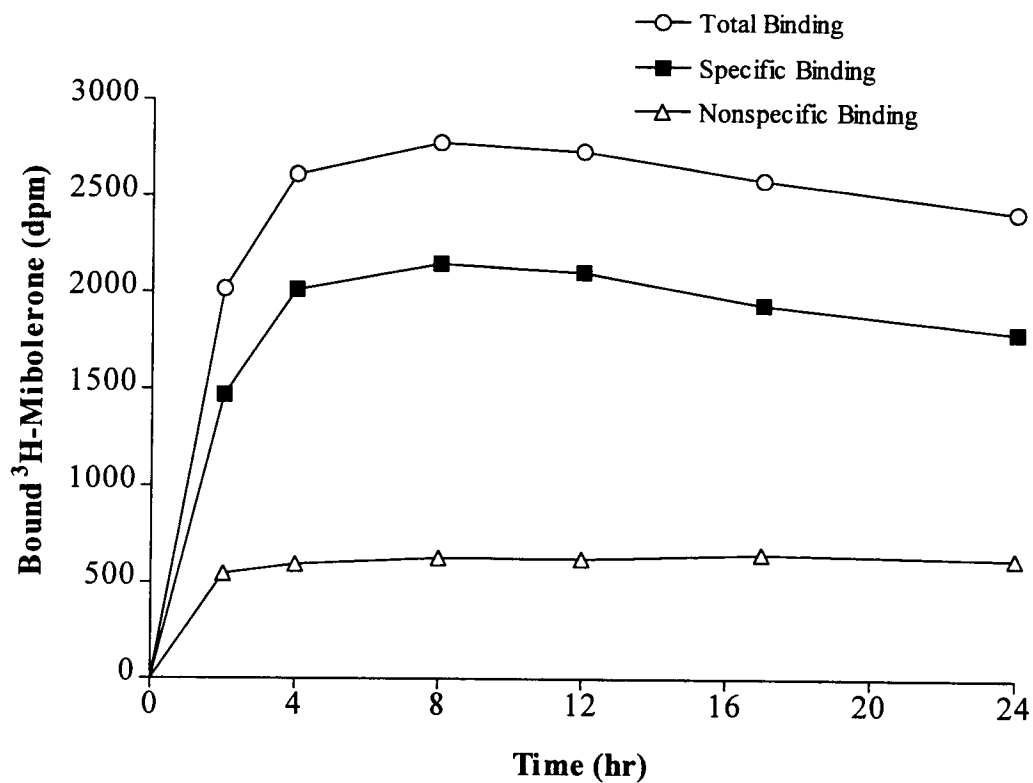


Figure 1.1. Association of  $^3\text{H}$ -Mibolerone (1 nM) and testicular cytosol at  $17^\circ\text{C}$ . Each point is the mean of three observations. Specific Binding was calculated as the difference between Total and Nonspecific Binding.

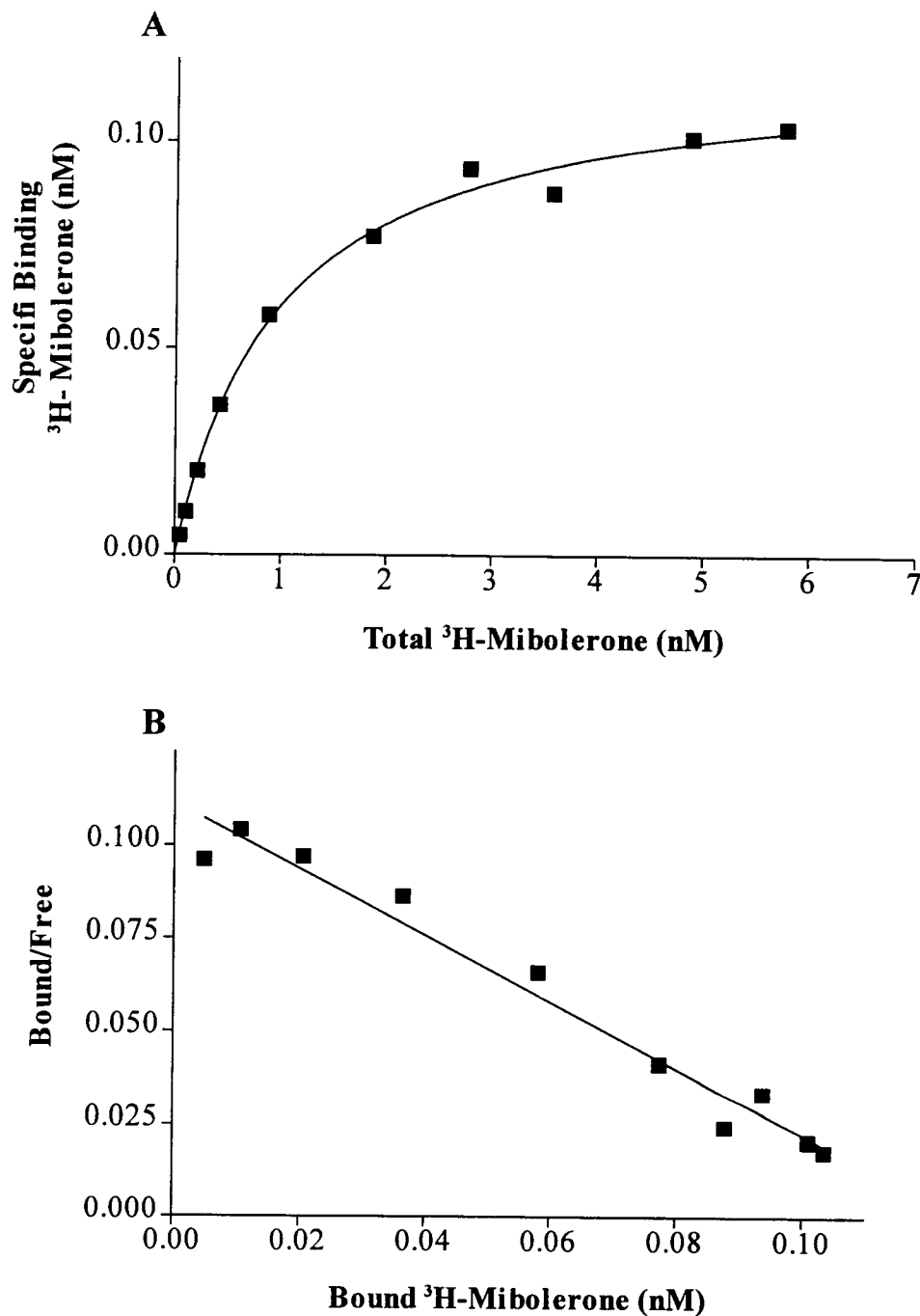


Figure 1.2. Saturation analysis of  $^3\text{H}$ -Mibolerone binding in testes. Cytosol was incubated for 12 hr at  $17^\circ\text{C}$  with varying concentrations of  $^3\text{H}$ -Mibolerone in the absence or presence of 100-fold excess radioinert mibolerone. A representative saturation curve is shown in (A). Data were analyzed using nonlinear regression. The  $K_d$  for this assay was  $1.0 \pm 0.1$  nM, and the  $B_{\text{max}}$  was  $39.9 \pm 1.1$  fmol/mg protein. Scatchard plot of the transformed data is given in (B). Data were analyzed using linear regression ( $r^2=0.97$ ).

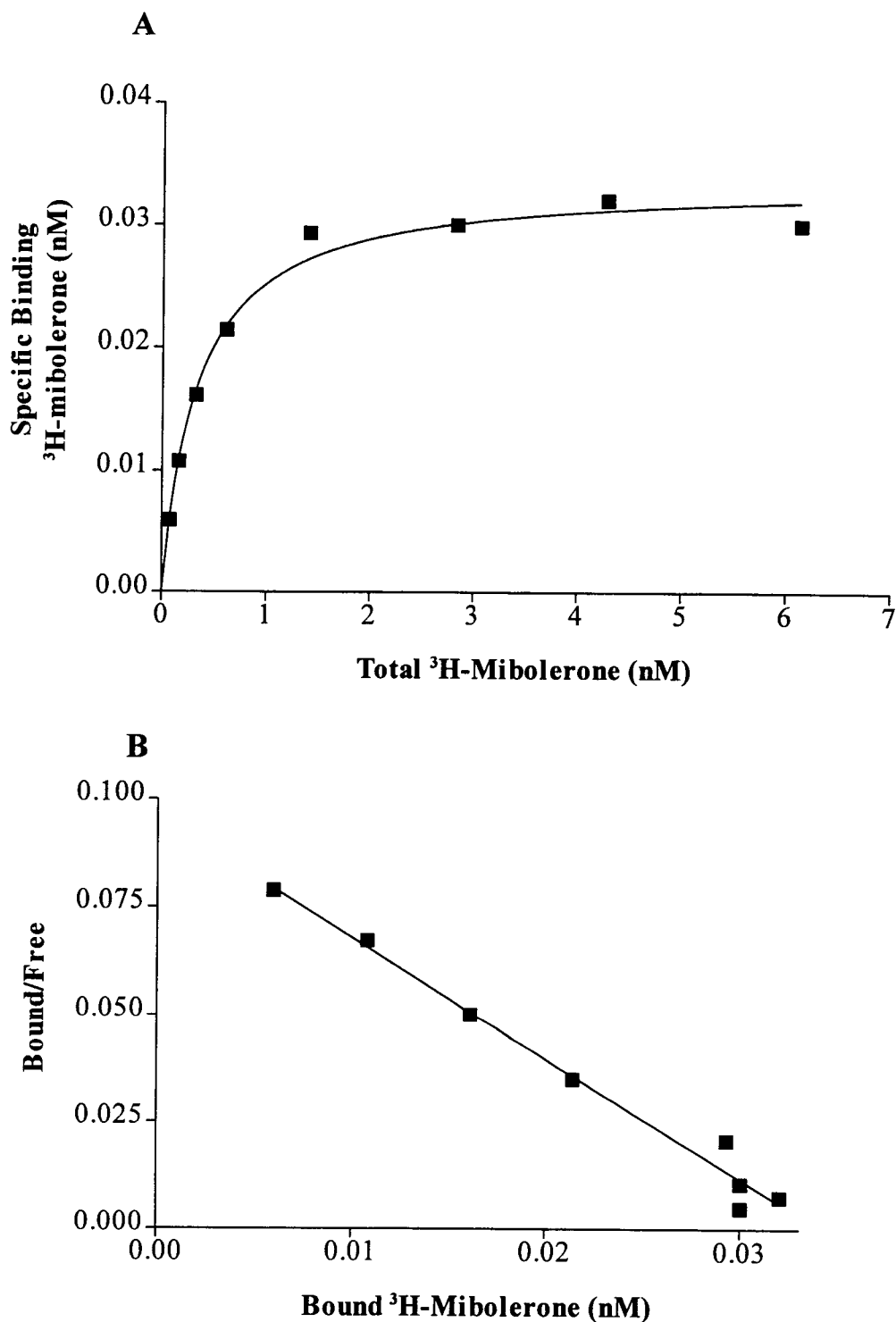


Figure 1.3. Saturation analysis of  $^3\text{H}$ -Mibolerone binding in immature ovaries. Experimental conditions and data analysis are as given in Figure 1.2. A representative saturation curve is shown in (A). The  $K_d$  for this assay was  $0.33 \pm 0.04$  nM, and the  $B_{\text{max}}$  was  $11.2 \pm 0.3$  fmol/mg protein. Scatchard plot of the transformed data is given in (B). The  $r^2$  of the fitted line was 0.98.

*Tissue specificity.*

Testicular and ovarian cytosol showed the greatest capacity to specifically bind  $^3\text{H}$ -Mb (Fig. 1.4). Specific binding was also measured in liver and heart cytosol, but it was considerably less than that observed in the gonadal tissues. Muscle and gill cytosol demonstrated no specific binding.

*Metabolism of mibolerone during incubation.*

Thin layer chromatography of  $^3\text{H}$ -Mb incubated with testicular cytosol revealed a single peak of radioactivity that migrated with authentic  $^3\text{H}$ -Mb (Fig. 1.5).

*Ligand specificity.*

Various steroids were assessed for their ability to displace  $^3\text{H}$ -Mb (Fig. 1.6) in testicular cytosol. The synthetic androgen MT was most effective at displacing bound  $^3\text{H}$ -Mb. Other compounds displaying strong competitive tendencies were DHT, KT, T, and EE. Progesterone,  $\text{E}_2$ , 17,20-DHP, and 11-OHAN were ineffective at displacing Mb. In immature ovarian cytosol,  $\text{E}_2$  and  $\text{P}_4$  were unable to displace  $^3\text{H}$ -Mb binding, whereas the synthetic androgens MT and MDHT were strong competitors for  $^3\text{H}$ -Mb binding (Fig. 1.7).

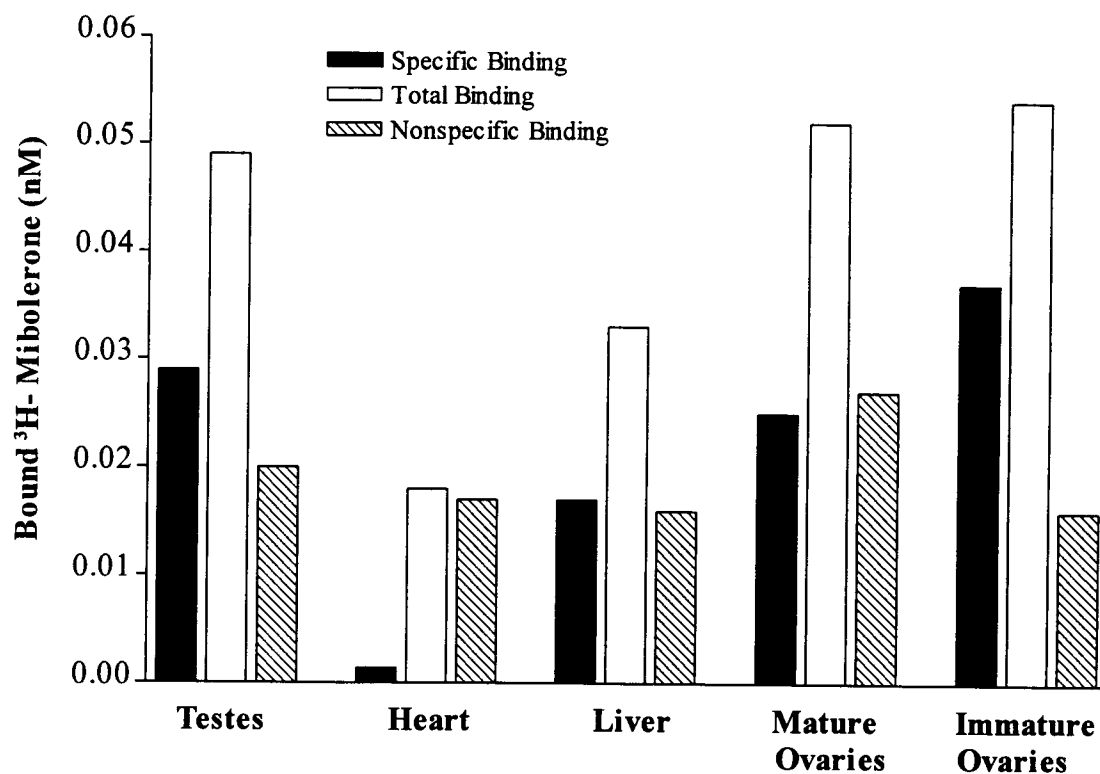


Figure 1.4. Binding of 1 nM  $^3\text{H}$ -Mibolerone to cytosolic extracts from different tissues. Cytosol was incubated for 12 hr at 17°C. Specific binding was calculated as the difference between Total and Nonspecific Binding. Each bar is the mean of three observations. Muscle and gill cytosol did not display specific binding (data not shown).

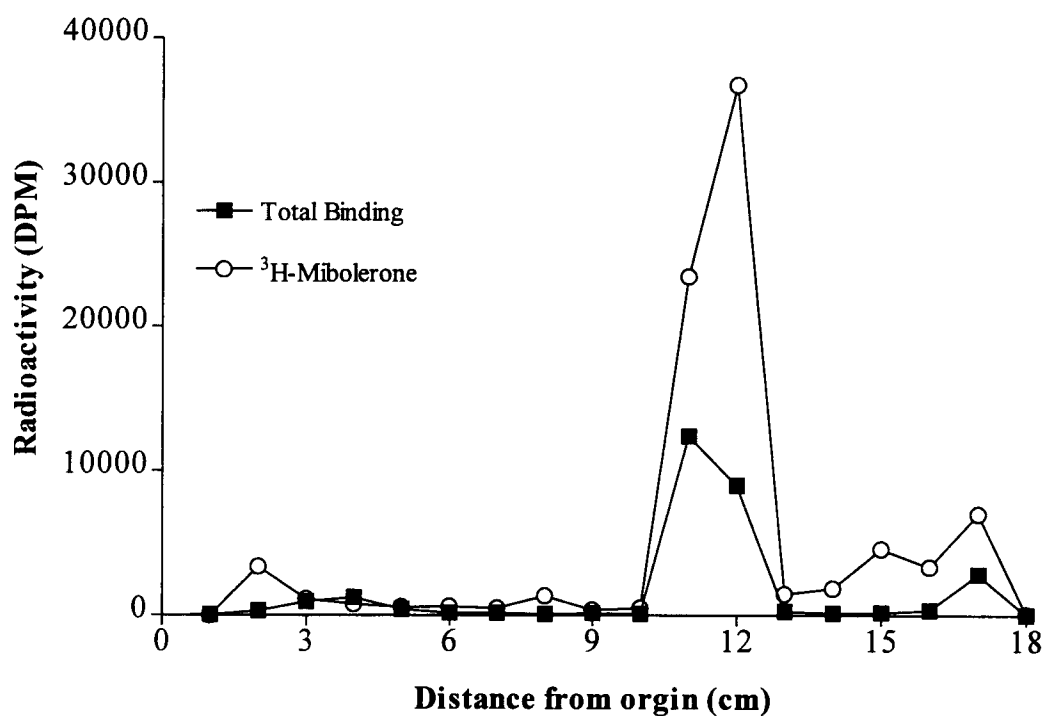


Figure 1.5. Thin layer chromatogram of ether extracted <sup>3</sup>H-Mibolerone following incubation with the testicular androgen receptor. Cytosol was incubated for 12 hr at 17°C with 1 nM <sup>3</sup>H-Mibolerone. Cytosol was then extracted with diethyl ether and subsequently chromatographed in dichloromethane:ether (5:2). Radiolabeled mibolerone was also chromatographed for use as a standard. Radioactivity was counted in 1-cm sections.

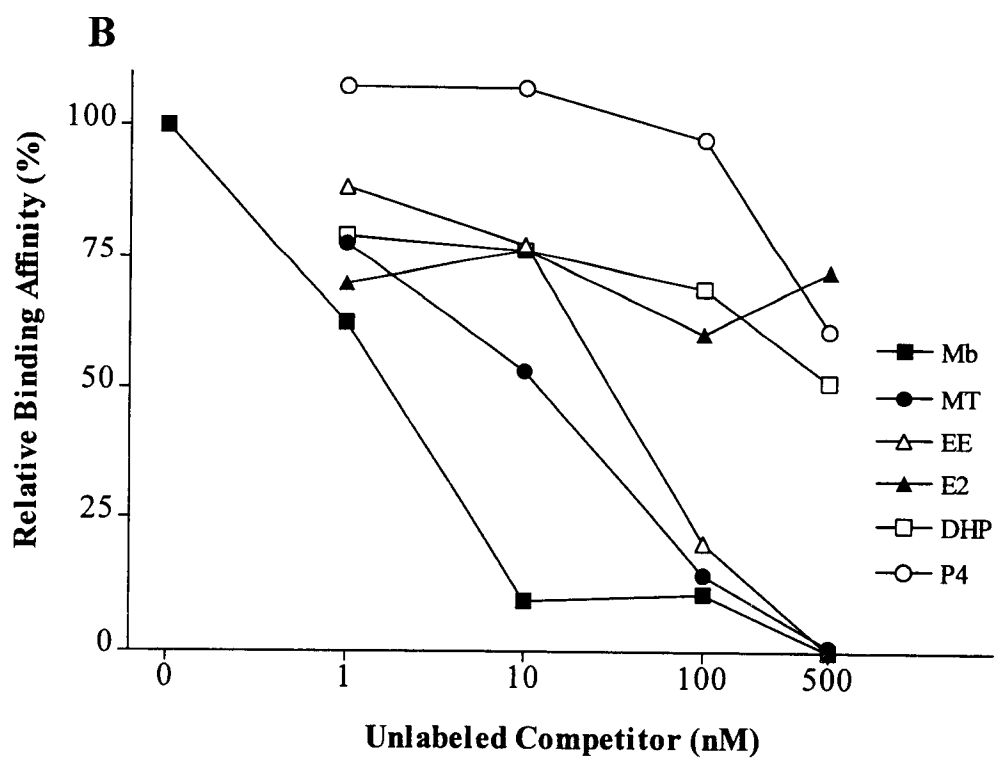
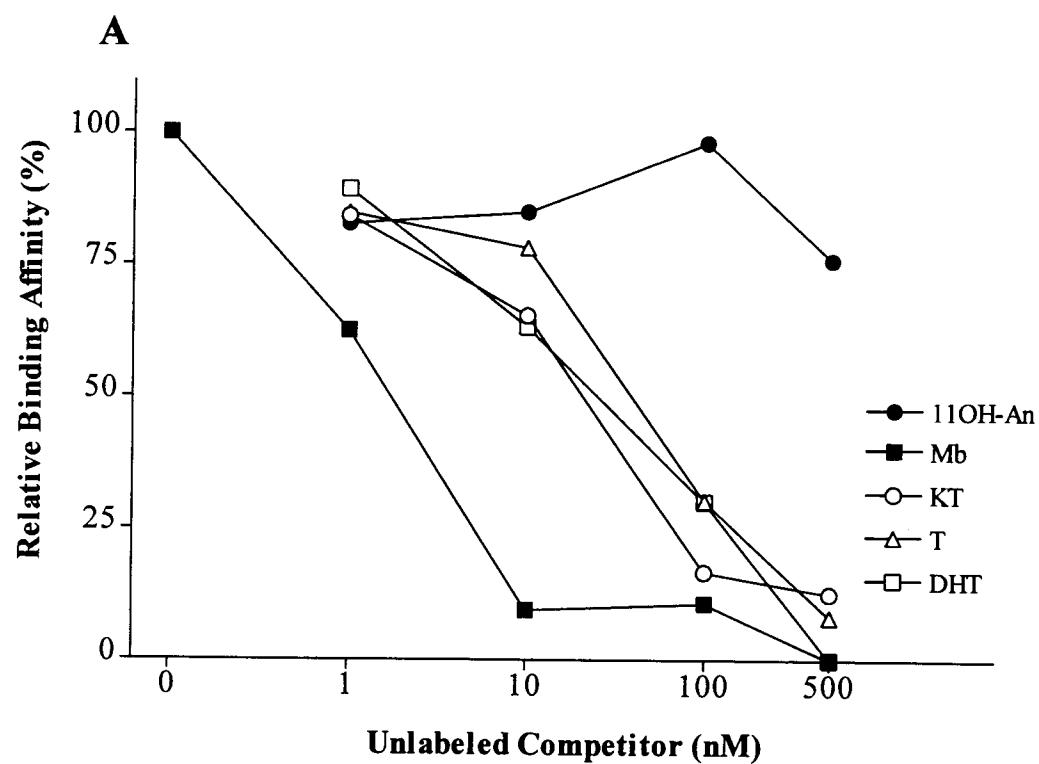




Figure 1.6. Competitive displacement of 1 nM 3H-Mibolerone from testicular cytosol by selected steroid competitors. Cytosol was incubated for 12 hr at 17°C. Relative Binding Affinity was calculated as the difference between Total Binding and binding in the presences of competitor, divided by the difference between Total Binding and the binding seen in the presence of 500 nM mibolerone (Mb). Each point is the mean of two to three observations. Naturally occurring androgen competitors are depicted in (A); synthetic steroid and non-androgen competitors are displayed in (B). Competitors used were: Mb, 11 $\beta$ -hydroxyandrostenedione (11-OHAn), 11-ketotestosterone (KT), testosterone (T), 5 $\alpha$ -dihydrotestosterone (DHT), 17 $\alpha$ -methyltestosterone (MT), 17 $\alpha$ -ethynylestradiol (EE), 17 $\beta$ -estradiol (E2), 17 $\alpha$ ,20 $\beta$ -dihydroxyprogesterone (DHP), and progesterone (P4).

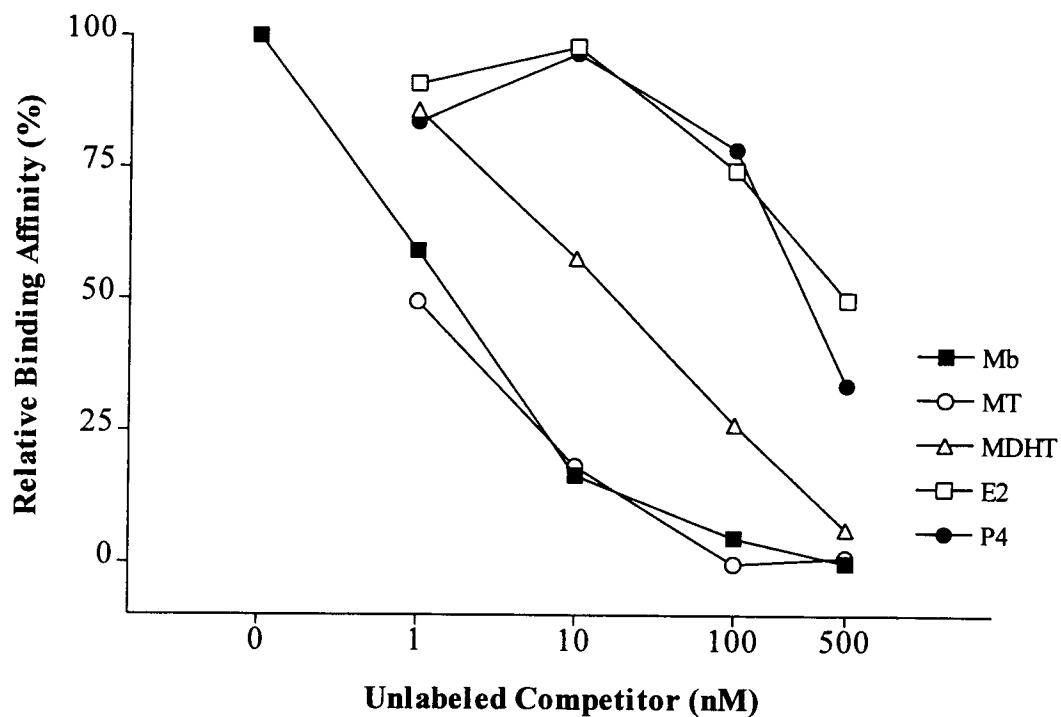


Figure 1.7. Competitive displacement of 1 nM  $^3\text{H}$ -Mibolerone from ovarian cytosol. Experimental conditions and data analysis are as described in Figure 1.6. Competitors used were: mibolerone (Mb),  $17\alpha$ -methyltestosterone (MT), methyl dihydrotestosterone (MDHT),  $17\beta$ -estradiol (E2), and progesterone (P4).

### *DNA-cellulose chromatography.*

A DNA-adhering fraction was detected after elution with 0.4 M NaCl buffer (Fig. 1.8). The radioactivity in this fraction was 33.0 % of the total specific binding in the incubation.

### Discussion

Our results support the hypothesis that an androgen receptor exists in the gonadal cytosol of Nile tilapia. The binding characteristics were consistent with the established criteria for steroid receptors (Clark and Peck, 1977). Saturation analysis revealed a saturable binding site in testicular cytosol that had high affinity for  $^3\text{H}$ -Mb ( $K_d=1.0\pm0.1$  nM) and low capacity ( $B_{\max}=35.8\pm1.2$  fmol/mg protein). Furthermore, the saturation data revealed that a single class of binding sites was responsible for the observed specific binding. Binding was tissue specific, as notable specific  $^3\text{H}$ -Mb binding was observed in only gonadal cytosol. The lack of binding in the nuclear preparations is unexpected, since most steroid receptors have been observed to be localized in the nuclear fraction of the cell (Carson-Jurica *et al.*, 1990). Absence of nuclear binding may be due to lack of nuclear binding sites or experimental conditions such as low protein levels (the highest protein concentration obtained in nuclear preparations was 2 mg/ml) or ineffective preparation of the nuclear extract.

The tilapia ovarian binding site had considerably different characteristics than the testicular AR. The ovarian site exhibited three-fold higher affinity ( $K_d=0.30\pm0.06$  nM) for  $^3\text{H}$ -Mb than did the testicular receptor. Furthermore, the number of ovarian receptors

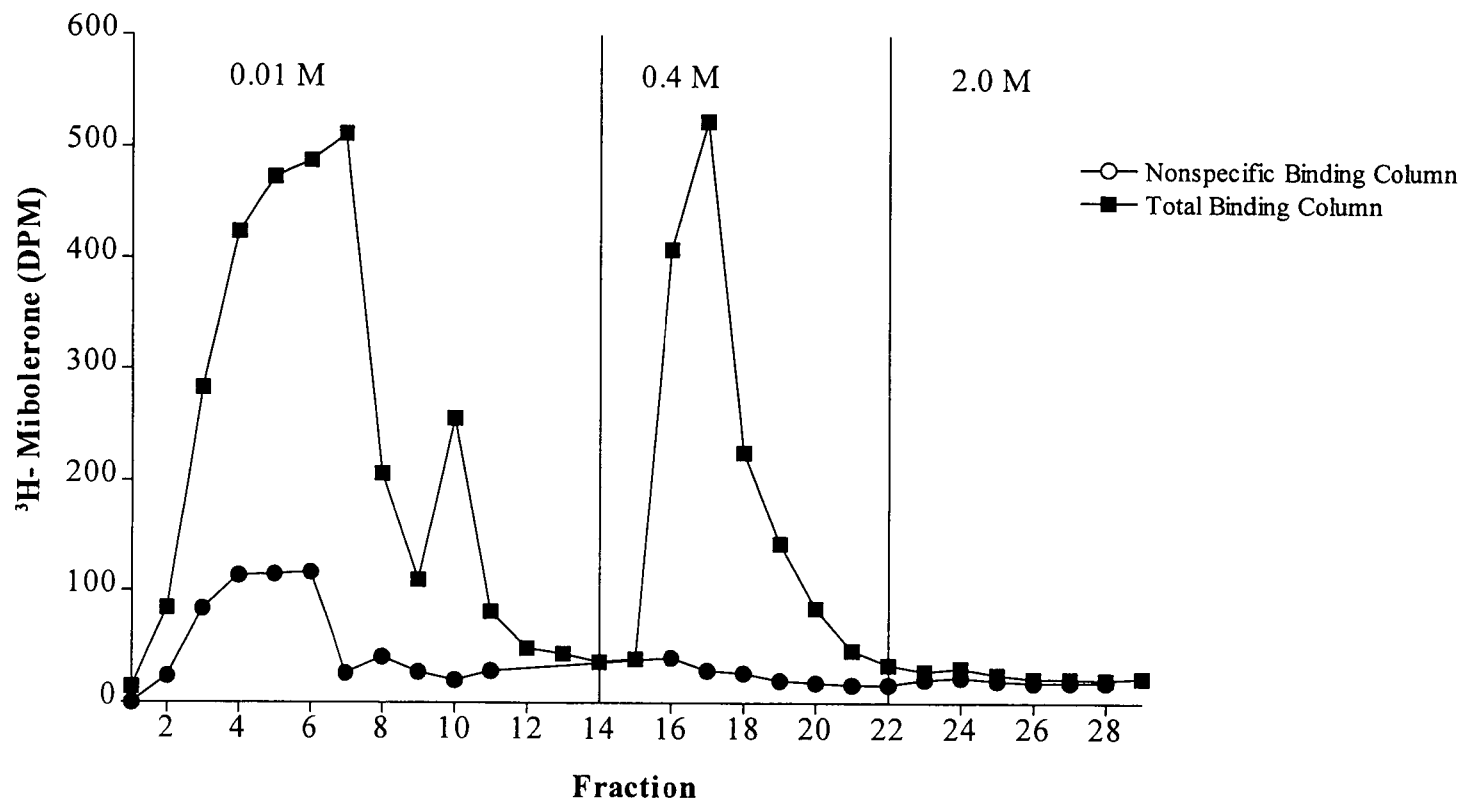


Figure 1.8. DNA-cellulose elution profile of the testicular androgen receptor. Cytosol was incubated at 4°C for 18 hr with 5 nM  $^3\text{H}$ -Mibolerone in the absence (Total Binding) or presence (Nonspecific Binding) of 100-fold excess radioinert mibolerone, then precipitated by addition of saturated ammonium sulfate solution, and diluted in molybdate free buffer. The steroid/receptor complex was incubated on the column for 30 min at 22°C and 30 min at 4°C. The column was washed sequentially with buffer containing NaCl at 0.01, 0.4, and 2 M, and radioactivity collected in 1 ml fractions.

( $14.6 \pm 1.1$  fmol/mg) was 2.5 times lower than the  $B_{\max}$  for the testicular site. This variation may reflect differences between the sexes in AR function and regulation.

The testicular AR in Nile tilapia exhibited specificity for androgens and sex-inverting steroids. Mibolerone masculinized *O. aureus* (Torrans *et al.*, 1988) at such low doses that the authors suggested that Mb may be one of the most potent masculinizing steroids yet tested. The strong masculinizing potency of Mb may explain its more acute displacement of  $^3\text{H}$ -Mb from the testicular AR in comparison to other masculinizing steroids. Of the other steroids tested, MT was the most effective competitor for  $^3\text{H}$ -Mb binding. It has long been used to masculinize undifferentiated fry of many teleosts (Schreck, 1974; Hunter and Donaldson, 1983). 11-Ketotestosterone also demonstrated an ability to displace  $^3\text{H}$ -Mb binding. This naturally-occurring steroid when implanted into ovariectomized adult female goldfish (*Carassius auratus*) caused growth of testicular tissue (Koybayashi *et al.*, 1991), it also masculinizes chinook salmon (*Oncorhynchus tshawytscha*; Piferrer *et al.*, 1993). The synthetic feminizing steroid EE reduced specific  $^3\text{H}$ -Mb binding to below 50%. This raises the possibility that steroid-induced feminization and masculinization may act through a common receptor, i.e., estrogen acts by competitive inhibition of the AR to cause feminization. Dihydrotestosterone, which also competed for Mb binding sites, has been shown to feminize channel (*Ictalurus punctatus*) and blue (*I. furcatus*) catfish, along with several synthetic androgens such as MT (Davis *et al.*, 1992). Two distinct scenarios may explain the displacement of  $^3\text{H}$ -Mb binding by DHT in tilapia. It is possible that DHT, acting as a feminizing agent, is

binding to the site in a manner similar to EE. Secondly, it is possible that DHT is a masculinizing agent in most other fish and that its feminizing effect in catfish is a paradox limited to *Ictalurus*. Given that DHT is an androgen and potently controls male sexual characteristics in most other vertebrate groups, the latter possibility is likely.

Fitzpatrick *et al.* (1994) observed ligand specificity for the ovarian AR in coho salmon similar to that which we described in the tilapia. Both the Nile tilapia and coho salmon AR display an equal or higher affinity for synthetic 17-alkylated androgens than for naturally-occurring androgens. If these receptors are present during sex differentiation, this unique ligand specificity would explain the greater sex-inverting potency of synthetic steroids as compared with natural steroids (e.g., MT compared with T). The ligand specificity of the tilapia AR and its gonadal location are consistent with the tilapia AR having a role in steroid-induced sex inversion.

Androgen receptors have been found in other fish but have considerably different qualities from the Nile tilapia AR. Such receptors have been described in the brain of goldfish (Pasmanik and Callard, 1988), the skin of brown trout (*Salmo trutta*; Pottinger, 1987, 1988), and in the testes of the spiny dogfish shark (Cuevas and Callard, 1992). The  $K_d$  for the testicular AR of the Nile tilapia is lower than that described for ARs in these other fish species, which reflects a higher affinity site. It must be noted that this  $K_d$  is for a synthetic steroid, whereas the previously described receptors used naturally-occurring steroids. Therefore, one might argue that the higher affinity is due to the use of a synthetic steroid as the ligand. However, synthetic steroids displayed a lower affinity

than that observed for naturally-occurring androgens, when used in other studies (Cuevas and Callard, 1992 and Pasmanik and Callard, 1988) with exception of the AR described by Fitzpatrick *et al.* (1994) in coho salmon. Clearly, the Nile tilapia AR is markedly different from most other androgen receptors in affinity and ligand specificity. It is likely that these characteristics are a result of differences in both form and function.

Nile tilapia ovarian binding sites have similar characteristics to the ovarian androgen receptor found in coho salmon (Fitzpatrick *et al.*, 1994). The  $K_d$  and  $B_{max}$  of the Nile tilapia ovarian binding site is comparable to the coho salmon AR ( $K_d=0.32\pm0.02$  nM,  $B_{max}=15.3\pm4.3$  fmol/mg protein; Fitzpatrick *et al.*, 1994). Furthermore, the ovarian binding sites of both coho salmon and Nile tilapia displayed similar ligand specificity. Binding sites of both species readily bound to 17-alkylated androgens and showed little affinity for  $E_2$  and  $P_4$ .

A clear role for androgens in the ovaries of fish is not established. Ovarian androgen binding sites may represent persistence of the receptor responsible for gonadal differentiation. Although androgens are thought to have little influence on oogenesis, it is possible that they are involved in this process. During oogenesis ovaries produce large quantities of androgens, and it is generally thought that these steroids are simply precursors for conversion to estrogen compounds (Scott *et al.*, 1983 and Fitzpatrick *et al.*, 1987). This scenario ignores the presence and ligand binding specificity of androgen binding sites in Nile tilapia and coho salmon.

Several roles are possible for the testicular AR in the tilapia. This receptor may be involved in spermatogenesis. A strong correlation between AR number and the initiation of spermatogenesis has been found in the spiny dogfish shark (Cuevas and Callard, 1992). Given the anabolic effects of androgens it is also possible that they are involved in testicular development and growth. Furthermore, the ligand specificity of the Nile tilapia AR strongly suggests a role in sex differentiation and steroid induced sex-inversion.

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## Chapter 2

### **Production of All-Male Populations of Nile Tilapia (Oreochromis niloticus) by Immersion in Methyldihydrotestosterone**

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### Abstract

The use of all-male populations increases the efficiency and feasibility of tilapia aquaculture. The objective of this study was to examine the efficacy of a short term immersion procedure for masculinizing Nile tilapia (Oreochromis niloticus). Two synthetic androgens were evaluated, methyl dihydrotestosterone and 17 $\alpha$ -methyltestosterone. Three hr exposures of fry at 10 and 13 days post-fertilization in methyl dihydrotestosterone at 500  $\mu$ g/l produced sex ratios greater than 93 percent male. Immersions in methyl dihydrotestosterone at 100  $\mu$ g/l and methyltestosterone at 500 or 100  $\mu$ g/l were unsuccessful at producing all-male populations. Immersion of Nile tilapia in 500  $\mu$ g/l methyl dihydrotestosterone may provide a practical alternative to the use of steroid-treated feed. Furthermore, when compared with current techniques for steroid-induced sex inversion of tilapia, short term immersion shortens the treatment period and reduces the risk of worker exposure to anabolic steroids.

Keywords: Sex Reversal; Oreochromis niloticus; Masculinization;

Methyl dihydrotestosterone

### Introduction

All-male populations are used in tilapia aquaculture because the culture of mixed sex populations often results in precocious maturation and early reproduction. Early maturation shunts energy to gonadal rather than somatic growth. Additionally, reproduction in ponds may lead to the harvest of many unmarketable fry. Individuals in mono-sex populations have increased somatic growth rate due to the avoidance of energy losses associated with gonadal development and reproduction. Furthermore, all-male tilapia populations are desirable because males achieve a larger final size than females.

One of the most common techniques for producing mono-sex populations is steroid-induced sex inversion. This involves the administration of synthetic androgens or estrogens to differentiating fry. The steroid acts as a sex-inversion agent by functionally masculinizing or feminizing individuals in the population. Several methods of steroid administration are possible, including injection, feeding of steroid, and immersion of fry in steroid solutions. Due to their non-invasive nature, the latter two are the most practical for application to aquaculture.

Use of steroid-treated feeds for the production of all-male populations is widespread in tilapia aquaculture. Conversely, use of immersion techniques is not fully developed for practical usage. Torrains et al. (1988) successfully masculinized blue tilapia (Oreochromis aureus) using a long-term, continuous immersion in the synthetic androgen mibolerone (Mb). Optimum conditions for treatment were a five-week immersion period ([Mb]=600 µg /l H<sub>2</sub>O) with steroid solutions replaced weekly. Pandian and Varadaraj

(1987) masculinized Mozambique tilapia (*O. mossambicus*) by immersion in  $17\alpha$ -methyl-5-androsten-3 $\beta$ -17 $\beta$ -diol (5 or 10  $\mu\text{g/l}$ ). The immersion period lasted 10 days, beginning at 10 days post-fertilization (DPF). Although the authors reported 100% masculinization, detailed information regarding temperature, type of culture system used, fish density, and frequency of water exchange during the immersion period was not included. These omissions make the replication and future application of this research difficult.

A potential problem encountered when developing new methods for steroid-induced masculinization is paradoxical feminization, which results in the inadvertent production of feminized rather than masculinized populations. This phenomenon is caused by the aromatization of the synthetic androgen to a feminizing estrogen compound (Piferrer and Donaldson, 1991). Paradoxical feminization can be avoided by use of nonaromatizable androgens (Piferrer and Donaldson, 1991).

The objective of this research was to develop a short term immersion procedure for the masculinization of Nile tilapia (*O. niloticus*). Two synthetic androgens were tested,  $17\alpha$ -methyltestosterone (MT;  $17\alpha$ -methyl-4-androsten-3-one) and methyl dihydrotestosterone (MDHT;  $17\alpha$ -methyl androstan-17 $\beta$ -ol-3-one). Methyl dihydrotestosterone is a  $17\alpha$ -methylated nonaromatizable derivative of dihydrotestosterone. Methyltestosterone is one of the most commonly used sex-inverting

agents, but is susceptible to aromatization and has been associated with paradoxical feminization in chinook salmon (Oncorhynchus tshawytscha; Piferrer and Donaldson, 1991).

### Materials and Methods

Steroids were obtained from Sigma Chemical Company (St. Louis, MO) and stored in stock solutions of HPLC grade methanol (10 mg/ml). Breeding families (one male to three females) were placed in 208 l aquaria. The temperature was maintained at 28-30°C. Breeding activity was monitored daily. Once breeding occurred, the other fish were removed and the brooding female left to incubate the progeny. At 10 DPF, fry were removed from the female and assigned to experimental groups (n=100/group). Groups of fry were housed in 3.8 l glass jars with 3 l of fresh water. The water was maintained at 28±2°C under constant aeration. Treatment consisted of a 3 hr immersion on 10 and again on 13 DPF. After immersion, the fry were collected and placed in new jars that contained fresh water. For each immersion treatment, steroid was evaporated under N<sub>2</sub> (g) and delivered in 0.5 ml of ethanol. Steroid was allowed to mix by aeration for 30 min before addition of fry. Fry were immersed in MT or MDHT at 100 or 500 µg/l (MT-100, MT-500, MDHT-100, MDHT-500). Control groups included the following: immersion in water and ethanol vehicle (ethanol group), an immersion in water alone (control

group), and water immersion followed by feeding of MT-treated diet (60 mg/kg) from 10 to 30 DPF. The MT-treated diet was made by dissolving steroid (30 mg) in 250 ml of 100% ethanol. The steroid solution was mixed with a commercial flake feed and allowed to dry before use. Other groups were fed commercial flake feed. Throughout the experiment fry were fed to satiation 3-5 times daily.

The first experiment was repeated (experiment two) with omission of the dietary MT control group. In experiment one, the groups were held in the jars (3.8 l) until the end of the feeding treatment period (30 DPF). In experiment two, fish were removed from the 3.8 l jars immediately following the 13 DPF immersion. Groups in both experiments were transferred to 20 l chambers for grow-out in a recirculating system. Water temperature in the grow-out system was maintained at  $28 \pm 2^\circ\text{C}$ . At 100 DPF, sex ratios were determined by examination of in situ (40X) and squash (100X) preparations after aceto-iron hematoxylin (Wittman, 1962) staining. Standard length and body weight of sampled fish was recorded in experiment two.

Sex ratio data were analyzed using the chi-square test ( $\alpha < 0.05$ ; Zar, 1984). The control and ethanol groups were not significantly different, and were pooled for comparison to other groups. Mortality data were analyzed using the chi-square test ( $\alpha < 0.05$ ; Zar, 1984). Since data were recorded for experiment two only, length and weight data were not analyzed statistically.



### Results

Immersion in MDHT at 500  $\mu\text{g/l}$  resulted in 100 (experiment one) and 94 (experiment two) percent male populations (Fig. 2.1). In the first experiment, MT and MDHT immersions at 100  $\mu\text{g/l}$  resulted in significant skewing of the sex distribution toward males (73 and 72 percent male, respectively). However, in experiment two, the proportion of males in these treatments were not significantly different from controls. Methyltestosterone at 500  $\mu\text{g/l}$  had no masculinizing effect in either experiment. The MT feeding treatment resulted in 92 percent males.

Immersion treatment did not significantly effect mortality in either experiment (Table 2.1). High mortality was seen in the control group from experiment one; this was associated with anoxic conditions caused by a clogged inlet during the grow-out period. The MT-500 group in experiment two suffered higher mortality due to cannibalization by an adult fish that jumped from an adjoining tank. Average final length and weight were similar between treatments (Table 2.2).

### Discussion

Immersion of Nile tilapia on 10 and 13 DPF with MDHT at a concentration of 500  $\mu\text{g/l}$  caused masculinization. Conversely, MT at similar levels did not significantly alter the sex ratio. Lack of an effect in the MT treatment (500  $\mu\text{g/l}$ ) may be due to conversion of MT to a less active form or simply a higher rate of clearance from the body than MDHT. Another possible explanation for the differing effects of the two steroids is that MDHT is a more potent masculinizing agent than MT. Piferrer et al. (1993) found that

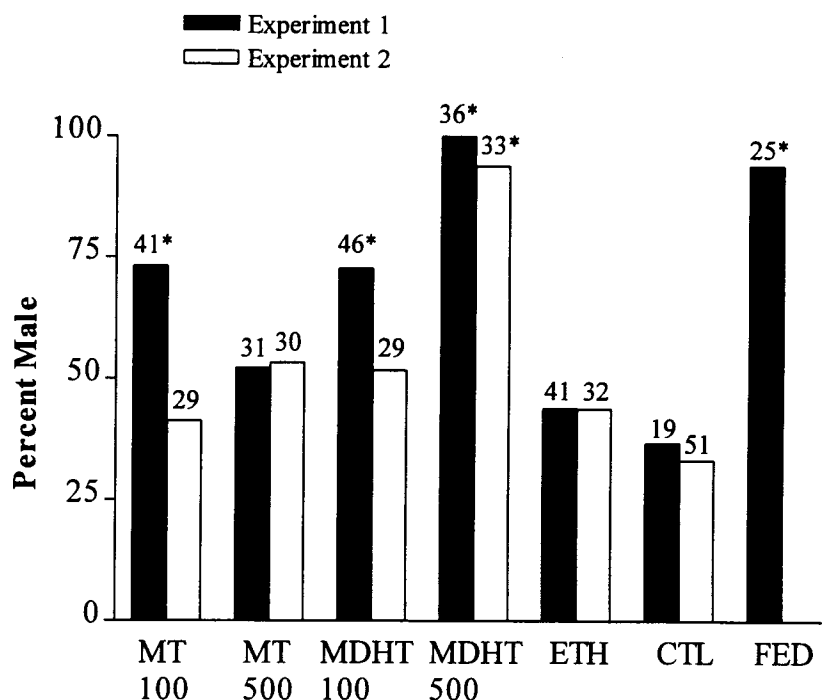


Figure 2.1. Percent males in each group for experiments one and two. Group designations are as follows: immersion treatment in 100 or 500  $\mu$ g 17 $\alpha$ -methyltestosterone/l (MT 100, MT 500), immersion in 100 or 500  $\mu$ g methyl dihydrotestosterone/l (MDHT 100, MDHT 500), immersion in ethanol vehicle (ETH), immersion in water alone (CTL), and methyltestosterone feeding treatment (FED) from 10-30 DPF (60 mg/kg feed). Asterisks indicate significant (from chi square test;  $\alpha \leq 0.05$ ) differences in proportion of males from the pooled control (ETH and CTL) group. Sample sizes are shown above bars.

Table 2.1. Mortality data for experiment one (EX 1) and two (EX 2). Group abbreviations and sample sizes are the same as given in Fig. 2.1.

	EX 1	EX 2
Group	Mortality (%)	Mortality (%)
MT-100	58	26
MT-500	46	64
MDHT-100	53	33
MDHT-500	63	33
ETH	59	22
CTL	81	35
FED	62	----

Table 2.2. Mean weight, and standard length ( $\pm$ SE) from sampled fish in experiment two. Group designations and sample sizes are the same as given in Fig. 2.1.

Group	Weight (g)	Length (mm)
MT-100	2.77 $\pm$ 0.21	41.7 $\pm$ 1.2
MT-500	3.45 $\pm$ 0.21	44.4 $\pm$ 1.0
MDHT-100	2.95 $\pm$ 0.30	41.7 $\pm$ 1.4
MDHT-500	3.29 $\pm$ 0.22	43.7 $\pm$ 1.1
ETH	2.97 $\pm$ 0.26	42.5 $\pm$ 1.3
CTL	3.17 $\pm$ 0.23	42.2 $\pm$ 1.0

MDHT was twice as potent as MT in masculinizing female chinook salmon.

Furthermore, MDHT can bind to androgen receptors in adult Nile tilapia (Gale, W.L., Fitzpatrick, M.S., and C.B. Schreck, unpublished results). These binding sites are specific for sex-inverting androgens, and are found in the gonadal cytosol.

Immersion treatment did not significantly affect mortality. Although mortality was not significantly different between treatments, fry in experiment one did suffer a higher mortality than did individuals in experiment two. This discrepancy is likely due to improvements in culture conditions. Fish in experiment one were held at a density of 33 fish/l for 20 days (30 DPF) and then placed in grow-out tanks at a density of 5 fish/l. To decrease mortality, fish in experiment two were held at the 33fish/l density for only 3 days (13 DPF) and then transferred into grow-out tanks at a density of 5 fish/l.

Administration of steroid by incorporation in feed has a long history of use (see reviews by Schreck, 1974 and Hunter and Donaldson, 1983). Steroid is dissolved in a carrier (e.g., ethanol or acetone), uniformly mixed with feed, and allowed to dry before use. Fry are fed for 20 to 35 days, beginning between 10 and 14 DPF. Although this technique usually results in successful sex inversion, certain inefficiencies are cause for concern. The dose received by an individual fish is variable--being dependent on body size, social status, and consumption of naturally-occurring food. This may result in an uneven distribution of steroid. The culturist must then accept partial or incomplete sex inversion or increase the treatment dose beyond the optimal requirement to achieve 100% sex inversion. Furthermore, the long period of treatment employed by typical feeding

methods results in human handling of anabolic steroid three to five times daily for up to 35 days. This degree of handling presents an added risk to the aquaculture worker, given the tumorigenic and teratogenic effects of anabolic androgenic steroids (Lewis and Sweet, 1993). This risk is easily mitigated by the establishment of proper handling procedures. However, these precautions are often improperly implemented. For instance, in developing countries where much of the worldwide tilapia production occurs, disposable rubber gloves for the handling of treated feed may be either unavailable or too expensive to be practical. Furthermore, in developing countries workers generally have little to no protective clothing (*e.g.*, rubber waders) for working in ponds containing dissolved steroid. Therefore, techniques that reduce worker exposure to anabolic steroid, but are as (or more) effective as feeding treatments need to be established.

The technique described in our study consisting of immersion in MDHT decreases the treatment period thereby reducing worker exposure while still achieving nearly complete masculinization. This technique is a promising alternative to the use of steroid-treated feed, but further evaluation is needed before application in large scale aquaculture operations.

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## Conclusions

A better understanding of steroid-induced sex inversion and gonadal differentiation has many possible benefits. To the aquaculturist, sex inversion offers a chance to improve yields and cut costs. Our immersion technique for sex inversion of Nile tilapia reduces the time needed for treatment, while also lessening the risk of worker exposure to anabolic steroids. This technique can be used to better define the window of sensitivity of fry to sex-inverting agents. Although the processes of steroid-induced sex inversion and sexual differentiation are not fully understood, it appears likely that steroids have a direct effect on the indifferent gonad in both processes. To mediate this proposed effect, specific steroid receptors are required. The tilapia AR may represent the molecule that facilitates androgen induction of gonadal differentiation and steroid-induced sex inversion. If this is true, the optimal period for sex-inversion would likely correspond with maximal receptor expression. Further purification of cytosolic preparations will allow generation of monoclonal antibodies specific to the tilapia AR. This will allow additional research examining the expression and function of ARs in differentiating fry. This work will further knowledge of gonadal differentiation and may also provide information key to increasing the efficacy of sex-inversion practices.



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